Separation of Sixteen Dinitrophenylamino Acids by Adsorption Chromatography on Silicic Acid–Celite

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In the determination of the free amino groups in proteins by the use of 2,4-dinitrofluorobenzene, a satisfactory method for isolation and identification of the dinitrophenylamino acids is essential. Because the success of the "partition" chromatographic method described by Sanger is highly dependent on the batch of silica gel used, a more reproducible method was desired. A systematic scheme is described for separation and identification of sixteen ether-soluble dinitrophenylamino acids by "adsorption" chromatography on silicic acid–Celite. A thorough investigation was made of the conditions necessary for obtaining reproducible results. The scheme was then tested on twelve batches of silicic acid from several sources; nine batches were entirely satisfactory without special treatment. The results indicate that the scheme is a relatively rapid method for isolating and identifying dinitrophenylamino acids and can be used on a wide variety of commercially available samples of silicic acid.

IN THE course of recent studies of the structure of proteins at the California Institute of Technology it became necessary to investigate the sequence of the amino acids in the polypeptide chains of certain proteins. At the time this work was started, the only method that had shown promise of yielding useful information was Sanger's method for the determination of free amino groups by the use of 2,4-dinitrofluorobenzene (*15*). In connection with this method he described a scheme for the separation and identification of the bright yellow 2,4-dinitrophenyl (DNP) derivatives of the amino acids by "partition" chromatography on silica gel columns. Sanger (*14–16*) and other authors (*2, 5, 9, 13*) have made it clear that the success of this method was highly dependent on the batch of silica gel used. In an attempt to resolve this difficulty Middlebrook (*9*) and Blackburn (*2*) modified the method by using phosphate-buffered columns of silica gel and reported that variations between different batches of gel were eliminated.

In addition to the above methods, a variety of other techniques for the separation of mixtures of 2,4-dinitrophenylamino acids have been described in the literature. Several brief papers have been published on the separation of 2,4-dinitrophenylamino acids by paper chromatography (*1, 3, 7, 10*), and a countercurrent extraction method for the separation of some 2,4-dinitrophenylamino acids and 2,4-dinitrophenylpeptides has been described (*24*). Recently two other "partition"-type methods have been described, one of which uses columns of chlorinated rubber (*11*) and the other buffered columns of Celite 545 (*12*).

Workers in another department at this institute had attempted to use Sanger's and Blackburn's methods on columns of silica gel prepared according to the procedure recommended by Sanger, but the results of their experiments had been discouraging. The preparation of the silica gel from water glass was laborious and the chromatographic properties of the gel were not very satisfactory.

Considerable success had been achieved in these laboratories with the use of "adsorption" chromatography on commercial silicic acid for the separation of nitrophenyl derivatives found in smokeless powder (*19–21*) and it was thought that this adsorbent might be used for the partition methods. Preliminary experiments with the partition method on commercial silicic acid adsorbent gave results similar to those obtained on the silica gel columns mentioned above. The zones formed on the unbuffered columns were broad and "tailed" badly. The differences in the rate of movement of the various derivatives were not so great as those reported by Sanger, so that the separations were poorer. On the buffered columns the derivatives moved about one fifth to one tenth as fast as expected. The rate of flow of the developing solvents was extremely slow, so that 10 to 24 hours were required to run each chromatogram.

Because adsorption chromatography on silicic acid–Celite had proved to be so useful for the separation of nitrophenyl derivatives in smokeless powder, it was thought that this technique might prove useful for the separation of the 2,4-dinitrophenylamino acids. Preliminary experiments indicated that the 2,4-dinitrophenyl derivatives were rather strongly adsorbed on prewashed silicic acid–Celite columns and formed compact zones which moved at a reasonable rate when developed with acetic acid in petroleum ether (ligroin). The development behavior of the 2,4-dinitrophenylamino acids was then studied with a variety of developers and a systematic scheme was devised for the qualitative separation and identification of sixteen ether-soluble 2,4-dinitrophenylamino acids. This method, which is described in the present paper, has the advantage of being much faster than the partition methods, as the solvents are pulled through the column by suction; an entire chromatogram can be run in from 0.5 to 2 hours. Furthermore, fewer chromatograms are required for the complete resolution of a mixture. When the scheme was tested on twelve batches of commercial silicic acid, nine of the samples gave satisfactory results, although there are slight variations in the adsorptive properties of the various samples.

The scheme has already been used successfully to determine the end group of the protein, lysozyme (*6*), and to analyze some peptides found in partial hydrolyzates of this protein (*18*).

EXPERIMENTAL PROCEDURES

2,4-Dinitrophenylamino Acids. The sixteen derivatives listed in Table I were prepared using 2,4-dinitrofluorobenzene in a manner similar to that described by Sanger for 2,4-dinitrophenylphenylalanine (*15*). All these derivatives were eventually obtained as solid products, although most of them came out initially as oils when the reaction mixture was acidified.

Suitable solvent systems for recrystallization were difficult to find and most of the samples were studied in the crude state. The crude samples gave only a single zone or else showed only negligible amounts of impurities. The melting point data for the derivatives are given in Table I.

Adsorbents. Except where otherwise noted, the silicic acid used in the chromatographic experiments was Merck reagent silicic acid 40446. The eleven other samples of silicic acid which were tested included three samples of Merck silicic acid identified by code numbers 43243, 42316, and 40665; one sample each from the General Chemical Co., New York; City Chemical Co., New York; and J. T. Baker Co. (Lot A-288), Phillipsburg, N. J.; and five samples from the Mallinckrodt Chemical Works, St. Louis,

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Table I. Melting Point Data on 2,4-Dinitrophenylamino Acids

DNP-Amino Acid	Recrystallized from	Melting Point, °C.	
DNP-DL-alanine	Acetone-ligroin	(167)[a]	172–3
DNP-DL-aspartic acid	Ethyl acetate–ligroin	(187)	188–189 (d)
Di-DNP-L-cystine	Acetic acid–water	(115)	118–121 (d)
DNP-DL-glutamic acid	(Crude product)	(149)	155–162
DNP-glycine	Acetone-ligroin	(189)	192–3 (d)
DNP-DL-isoleucine	Acetone-ligroin		168–172
DNP-DL-leucine	Acetone-ligroin	(130.5)	131.5–132.5
Di-DNP-L-lysine	Formic acid; methanol–water	(170)	173–174.5
DNP-DL-methionine	Ether-ligroin	(104)	107–112
DNP-DL-phenylalanine	Acetone-ligroin	(199)	204–6 (d)
DNP-L-proline	Ether-ligroin	(134)	137–138.5
DNP-DL-serine	Acetone-ligroin	(185)	186–188 (d)
DNP-DL-threonine	Acetone-ligroin; ether-ligroin	(175.5)	177–178
DNP-L-tryptophan	(Crude product)	(195)	196–8 (d)
Di-DNP-L-tyrosine	(Crude product)	(84)	92–98 (d)
DNP-DL-valine	Acetone-ligroin	(180)	182.5–183.5

[a] Figures in parentheses indicate point at which some shrinkage of sample began; samples appeared to contract and occasionally a few beads of liquid appeared on the surface; then for a short interval before melting no further visible change occurred.

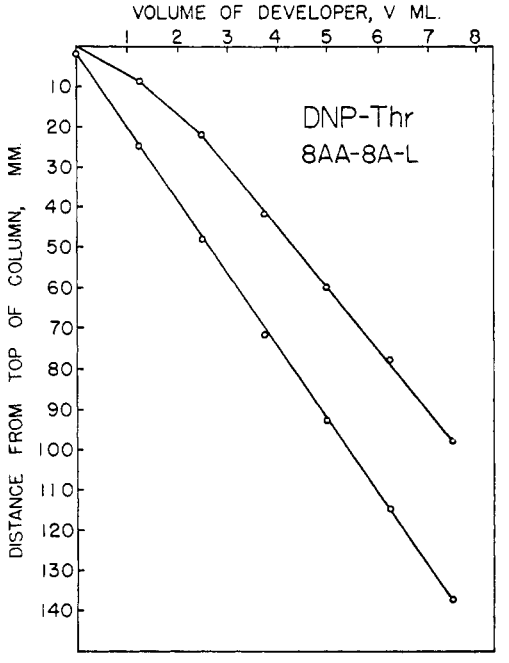

Figure 1. Typical Plot of Development Behavior of 2,4-Dinitrophenylamino Acids on Prewashed Silicic Acid–Celite

2,4-Dinitrophenylthreonine developed with 8AA-8A-L

Mo., listed as XAP, RCT-1, XMH-1, XXE-1, and XXG-1. The latter three samples were labeled by the manufacturer as "specially prepared for chromatographic analysis." The code numbers of the Merck samples are reported to refer to the packing date and presumably designate different production lots. In most experiments the silicic acid was thoroughly mixed with 0.5 part by weight of the filter aid, Celite 545, a product of the Johns-Manville Co. The Celite had been passed through a 60-mesh sieve to remove large particles. In a few specified cases the weight ratio of silicic acid to Celite was increased to 4 to 1.

Solvents. Reagent grade formic and acetic acids, acetone, ethyl acetate, and anhydrous ether, C.P. grade benzene, Skellysolve B ligroin (60–70°), and Matheson Co. white label cyclohexane were used. It is recommended that all the solvents be redistilled, although this was not always done in the experiments described. Because water has a considerable effect on the development behavior of the 2,4-dinitrophenylamino acids, care should be exercised to keep the reagents dry.

General Chromatographic Procedure. CHROMATOGRAPHIC TUBES. All the chromatograms were carried out in borosilicate glass chromatographic tubes of the type described by Zechmeister and Cholnoky (*25*). The tubes were about 200 mm. long; the columns of adsorbent after packing were 150 ± 5 mm. in height. Most of the experiments were carried out in tubes with inside diameters of 9 or 14 mm. but tubes 5 mm. in diameter were sometimes used for small amounts of the derivatives. The amount of 2,4-dinitrophenylamino acid which can be placed on the various sizes of tubes is discussed in a later portion of this paper.

PACKING OF COLUMNS. The columns were packed in a manner similar to that described by Trueblood and Malmberg (*23*). A partial vacuum was maintained by applying the full suction of a water aspirator to the system and allowing a sizable leak through a stopcock in the line. A small cotton plug was placed on the perforated plate at the base of the tube, the adsorbent was poured into the tube until it was filled, and the stopcock was then closed so that the full suction of the aspirator was applied. After the column had contracted to a constant height, the top surface was carefully smoothed in order to minimize distortion of the zones. Extreme care was also exercised in pouring the solvents onto the column, so that the surface was disturbed as little as possible. After the column had been packed, the suction was maintained either by continued use of the aspirator or by shifting to a suction pump maintaining a pressure of 70 to 80 mm.

PREWASHING PROCEDURE. Except where otherwise noted, the columns were prewashed by successively passing through the column 0.2 *V* ml. of ether, *V* ml. of 1 to 1 acetone-ether, 0.8 *V* ml. of ether, *V* ml. of ligroin, and *V* ml. of the developing solvent (see Table II for definition of *V* ml.). This procedure, which is similar to the "acetone-ether" prewash mentioned by Trueblood and Malmberg (*22*), removes essentially all the "free" water from the adsorbent and has the effect of decreasing the width of the zones and rendering the adsorbent more uniform throughout the column. However, contrary to the usual observations (*17*) with the 2,4-dinitrophenylamino acids the adsorptive strength of the adsorbent is decreased rather than increased by the prewash.

Table II. Abbreviations Used

AA = acetic acid	A = acetone	L = ligroin
F = formic acid	E = ethyl acetate	B = benzene
		C = cyclohexane

DNA = 2,4-dinitroaniline

V ml. = volume of solvent required to wet completely a column of adsorbent (*17*)

$$R \text{ value} = \frac{\text{linear rate of movement of zone}}{\text{linear rate of movement of solvent}} \quad (8)$$

Abbreviations for amino acids follow Brand's suggestions (*4*)

PLACING SAMPLE ON COLUMN. As the last of the prewashing solvents passed into the adsorbent, the sample, in about 1 to 3 ml. of a suitable solvent, was placed on the column and the walls of the tube were rinsed with developing solvent as the last of the sample solution passed into the adsorbent. In order to minimize distortion of the zones, the columns should never be allowed to become dry while the solvents are being passed through. For most of the chromatograms in the scheme the sample can be placed on the column in acetone-ligroin. The sample solutions are made up by dissolving the material to be chromatographed in acetone and then adding ligroin until the solvent has the desired composition. For the more soluble derivatives 10% by volume of acetone in ligroin (10A-L) is sufficient, but for the least soluble as much as 20% of acetone (20A-L) may be necessary. However, the 2,4-dinitrophenylamino acids are usually more soluble in acetic acid than in acetone and where some material precipitates out when ligroin is added, it may be necessary to evaporate off the solvents, take the residue up in acetone plus a few drops of acetic acid, and again add ligroin. In such cases the sample solvent must not be stronger than the developing solvent to be used and the volume must be kept small.

DEVELOPING OF ZONES. As the last of the sample solution passes into the adsorbent, the walls of the tube are washed with small amounts of the developing solution. The developer is then passed through the column until the zones have reached the desired positions. After each *V* ml. of developing solvent has passed into the column, the positions of the zones are recorded. A typical plot of the development behavior of the 2,4-dinitrophenylamino acids is shown in Figure 1.

In this paper the developing solvents are referred to by a series of numerals and letters which indicate their composition. The

numerals refer to volume percentages and the letters to the component solvents (see Table II). For example, developer 8AA-4A-L is prepared by placing 8.0 ml. of acetic acid and 4.0 ml. of acetone in a graduated cylinder and filling the cylinder to 100.0 ml. with ligroin.

The strengths of the developers for a given group of similarly adsorbed derivatives were usually chosen so that 5 to 7 V ml. would move the group to the lower two thirds of the column—that is, so that the R values of the derivatives were about 0.1. If stronger developers were used, the zones tended to spread out and were poorly separated.

Treatment of Zones after Development. Whenever it was convenient, development was stopped while all the zones were still on the column. In the experiments to determine the development behavior of the individual 2,4-dinitrophenylamino acids, the adsorbent was extruded from the tube and sliced in half axially, and the position of the zone on the axis was measured. However, if it was desired to study a zone further, the column was extruded and the section of the column containing the zone was cut and eluted with a 1 to 4 mixture of ethanol and ether. In some instances where it was necessary to wash zones off the column, the filtrate was collected in fractions which could be prepared for rechromatographing. The solvent was then evaporated from the eluates or filtrates under vacuum below 40° C. and the residue was taken up in a solvent suitable for placing it on a column.

Factors Affecting Development Behavior. Effect of Composition and Volume of Sample Solvent. The effect of the composition and volume of the sample solvent was determined for 2,4-dinitrophenylvaline, one of the least strongly adsorbed derivatives, and for 2,4-dinitrophenylglutamic acid, one of the most strongly adsorbed. It was found that if samples were placed on the column in acetone-ligroin, the volume of solvent was not too critical, but if the sample solvent was also a good developer—e.g., if it contained acetic acid—the volume had to be kept relatively small to avoid spreading of the zones.

When 2,4-dinitrophenylvaline was placed on 9-mm. diameter columns in from 0.5 to 8 ml. of 10A-L and developed with 4AA-2A-L, the width of the initial zone and the subsequent development behavior were essentially the same regardless of the volume of the solvent. When samples were placed on the column in 2 ml. or less of 4AA-2A-L, the behavior was the same as with 10A-L, but if the sample volume was increased above 2 ml., the width of the zone also increased. In similar experiments in which 2,4-dinitrophenylglutamic acid was placed on columns in 20A-L and developed with 8AA-8A-L, all the zones were essentially identical, but when this derivative was placed on columns in 5AA-B and developed with 5AA-B, the width of the zones increased as the sample volume increased.

Effect of Amount of 2,4-Dinitrophenylamino Acid. Experiments in which various amounts of 2,4-dinitrophenylvaline, 2,4-dinitrophenylglycine, and 2,4-dinitrophenylglutamic acid were placed on the columns showed that there is little visible change in the width of the zones and in their behavior over a rather wide range. However, the color intensity increases with the size of the sample, and better separations are obtained when the columns are not "overloaded." On the other hand, because some of the derivatives give much paler zones than others, the sample must be large enough to be visible. On a 9-mm. diameter column the samples should contain about 0.1 to 0.5 mg. of each derivative to obtain the best results, although as little as 0.03 mg. can often be detected. On a 5-mm. column, 0.01 mg. of the 2,4-dinitrophenylamino acids gives good zones, and much smaller quantities are still visible.

Effect of Water. The presence of a small amount of water in the system causes at least some of the 2,4-dinitrophenylamino acids to be more strongly adsorbed. This fact has been illustrated by experiments with various prewashing procedures and by deliberately adding water to the developers.

When a mixture of 2,4-dinitrophenyltryptophan and 2,4-dinitrophenylglycine was developed on a column which had been prewashed only with ether followed by ligroin and developer, the zone moved through the upper 25 mm. of the column at the same rate as on columns that had received the acetone-ether prewash recommended in this report. However, after the zone left the top 25 mm., the rate of movement decreased markedly. 2,4-Dinitrophenylvaline developed with 4AA-2A-L behaved similarly. This phenomenon was undoubtedly due to the fact that the "free" water had been removed completely from only the top portion of the column. When only ligroin and developer were used to prewash a column, the rate of movement was slower throughout the entire chromatogram. These results are contrary to the usual observation that the acetone-ether prewashing procedure increases the adsorptive strength of the column (*17*).

To determine the effect of small amounts of water in the developing solvents, 2,4-dinitrophenylserine, 2,4-dinitrophenylthreonine, 2,4-dinitrophenylaspartic acid, and 2,4-dinitrophenylglutamic acid were developed with 8AA-8A-L which had been prepared with acetic acid to which about 1.0% of water had been added. The rate of movement was 25 to 30% less than with dry 8AA-8A-L. The derivatives of the dicarboxylic amino acids were affected more than those of the hydroxyamino acids. 2,4-Dinitrophenyltryptophan developed with 8AA-4A-L exhibited similar effects. It appears probable that all the 2,4-dinitrophenylamino acids are affected to some extent by the presence of water.

Table III. Relative Order of Adsorption Affinities of 2,4-Dinitrophenylamino Acids with Various Solvent Systems

	Acetic Acid and Acetone in Ligroin	Acetic Acid and Ethyl Acetate in Ligroin	Acetic Acid in Benzene
Group I	Di-DNP-cystine	Di-DNP-cystine	Di-DNP-cystine
	DNP-serine	DNP-serine	DNP-serine
	DNP-aspartic acid	Di-DNP-lysine	DNP-threonine
	Di-DNP-lysine	DNP-threonine	DNP-aspartic acid
	DNP-threonine	DNP-aspartic acid	DNP-glutamic acid
	Di-DNP-tyrosine	Di-DNP-tyrosine	Di-DNP-lysine
	DNP-glutamic acid	DNP-glutamic acid	Di-DNP-tyrosine
Group II	DNP-tryptophan	DNP-glycine	
	DNP-glycine	DNP-tryptophan	
Group III	DNP-proline	DNP-proline	
	DNP-alanine	DNP-alanine	
	Dinitroaniline	Dinitroaniline	
	DNP-methionine	DNP-methionine	
	DNP-phenylalanine	DNP-phenylalanine	
Group IV	DNP-valine	DNP-valine	
	DNP-isoleucine	DNP-isoleucine	
	DNP-leucine	DNP-leucine	
	Dinitrophenol	Dinitrophenol	

Effect of Rechromatographing. Experiments have been carried out to determine whether a given derivative, after being chromatographed once, would still have chromatographic properties similar to a sample that had not been chromatographed previously. Several of the 2,4-dinitrophenylamino acids were chromatographed, eluted, and rechromatographed. The rate of movement was sometimes decreased slightly.

Effect of Hydrolysis Procedure. To determine how the behavior of the derivatives would be affected by the hydrolysis procedure used in releasing the 2,4-dinitrophenylamino acids from 2,4-dinitrophenylproteins, a sample of 2,4-dinitrophenylserine and 2,4-dinitrophenylthreonine was refluxed for 24 hours with 6 N hydrochloric acid. The reaction mixture was cooled and extracted four times with 25 ml. of ether. The residue left by the evaporation of the combined ether extracts was chromatographed with 8AA-8A-L and its behavior was compared with that of an untreated mixture of the two derivatives. The material which had been "hydrolyzed" moved slightly slower than the untreated and gave a small amount of a third zone which behaved like dinitroaniline with this developer but was not investigated further.

Table IV. Separation of Pairs of Derivatives in Groups I and III

Developer	6AA-12A-L	8AA-8A-L	10AA-5A-L	8AA-15E-L	10AA-10E-L	5AA-B
			Group I			
DNP-ser, -thr	**	**	**	o	**	*
DNP-ser, -asp	o	o	o	**	**	**
DNP-ser, -glu	**	**	**	**	**	**
DNP-ser, di-lys	**	**	o	o	*	**
DNP-ser, di-tyr	..	**	**	**	**	**
DNP-thr, -asp	o	*	o	o	o	**
DNP-thr, -glu	c	o	o	**	**	**
DNP-thr, di-lys	c	o	o	o	o	**
DNP-thr, di-tyr	..	o	c	**	o	**
DNP-asp, -glu	o	**	**	o	*	o
DNP-asp, di-lys	o	**	c	**	o	**
DNP-asp, di-tyr	..	**	o	o	o	**
DNP-glu, di-lys	c	o	**	**	**	**
DNP-glu, di-tyr	..	c	o	c	o	**
di-DNP-lys, -tyr	..	o	o	**	o	**
	2AA-8A-L	4AA-4A-L	8AA-4A-L	3AA-12E-L	5AA-5E-L	2F-8E-L	2AA-6A-C
			Group III				
DNP-pro, -ala	**	o	*	**	*	**	o
DNP-pro, -met	**	*	**	**	**	**	**
DNP-pro, -phe	**	**	**	**	**	**	**
DNP-pro, DNA	..	..	*	**	*	**	**
DNP-ala, -met	c	o	o	o	o	o	*
DNP-ala, -phe	c	*	o	**	**	**	**
DNP-ala, DNA	..	..	o	o	c	**	**
DNP-met, -phe	c	o	o	o	**	**	o
DNP-met, DNA	..	..	o	o	o	**	o
DNP-phe, DNA	..	..	o	*	**	o	o

** = fair to good separation.
* = two zones visible but interzone still colored.
o = zones overlap.
c = zones coincide.
.. = no data.

CHROMATOGRAPHIC BEHAVIOR OF 2,4-DINITROPHENYLAMINO ACIDS

Chromatographic Behavior with Various Developers. Some acid such as formic or acetic acid is necessary in order to obtain satisfactory zones. With acetone, ethyl acetate, or propanol in ligroin the zones spread down the column but the upper edge did not move more than a few millimeters from the top. When even a small amount of nitromethane was present in the sample solvent or developer, narrow, sharply defined zones were obtained but all the derivatives moved at approximately the same rate.

Acetic acid in ligroin gave good, compact zones and was a satisfactory developer for the less strongly adsorbed 2,4-dinitrophenylamino acids. However, acetic acid is miscible with ligroin only in mixtures containing about 6 volume % or less of the acid and this mixture was not a strong enough developer for many of the 2,4-dinitrophenylamino acids studied. By adding acetone or ethyl acetate as well as acetic acid to the ligroin, much stronger and more versatile developers could be prepared. Developers containing formic acid gave zones which were even more compact than those obtained with acetic acid. However, formic acid is not very soluble even when acetone or ethyl acetate is present, so that it is far less useful as a component in developers.

The preliminary studies with 6AA-L as developer indicated that certain groups of the derivatives have similar adsorption affinities and are separable from one another. The studies with acetic acid and acetone or ethyl acetate in ligroin have shown that with almost any of these solvent systems the derivatives fall into the same groupings, although some of the developers separate the groups more effectively than others.

The individual members of each group were studied with developers in which the volume of acetic acid was less than, equal to, and greater than the volume of acetone or ethyl acetate, in order to determine how such variations affect the relative adsorption affinities of the derivatives. In cases where satisfactory separations of some of the derivatives could not be obtained with mixtures of acetic acid and acetone or ethyl acetate in ligroin, other solvent mixtures were tried. The results of these studies are shown in Figure 2. In the figure the heavy bars represent the final positions of the various derivatives after development with the amounts and types of developers given at the bottom of each column. Such diagrams give a much clearer picture of the relative positions and possible separations than does a table of R values. The figure shows how the relative adsorption affinities can be altered by merely changing the ratio of acetic acid to acetone or ethyl acetate—for example, 2,4-dinitrophenylaspartic and 2,4-dinitrophenylglutamic acids, which coincide with 6AA-12A-L, can be separated with 8AA-8A-L. Figure 2 also shows that the relative order of adsorption affinity for the members of Groups I and II is changed when ethyl acetate rather than acetone is used in the developer or when acetic acid

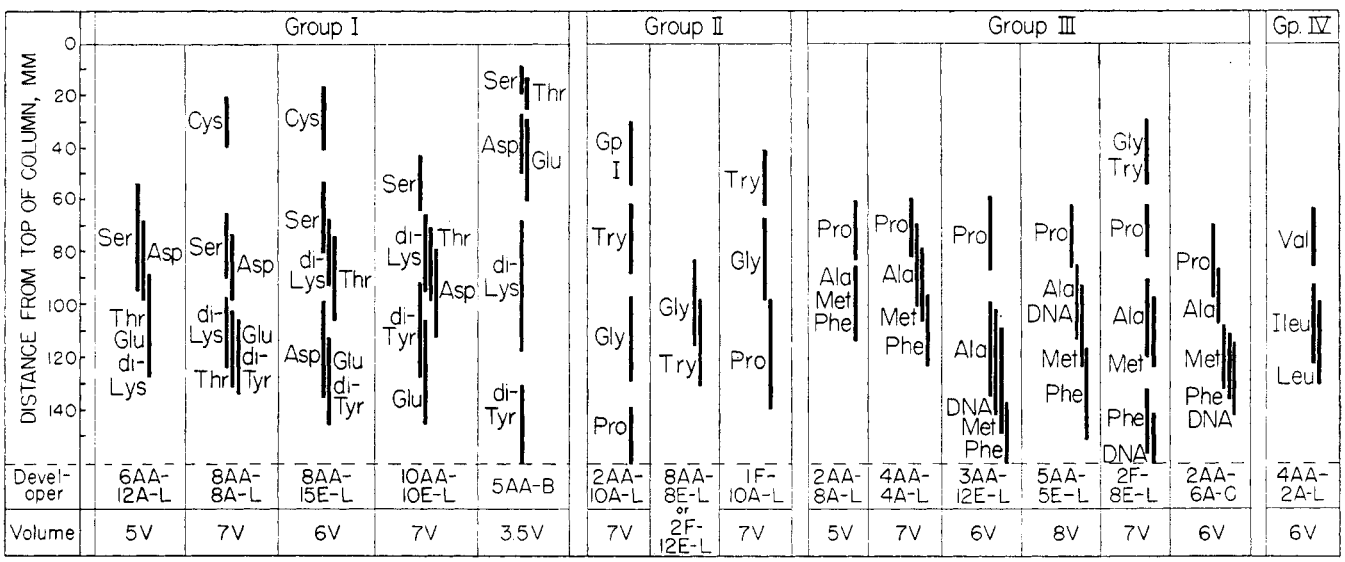

Figure 2. Relative Position of Dinitrophenylamino Acids After Development with Various Solvent Systems on Prewashed Silicic Acid (Merck 40446)–Celite

in benzene is the developer. The relative order of adsorption of the 2,4-dinitrophenylamino acids with several solvent systems has been determined from these data and is given in Table III.

The members of Groups III and IV are in the same relative order with all the solvent systems that have been tried. The relative order of the 2,4-dinitrophenylamino acids is essentially the same as that reported by Sanger (*15*).

If the data for two similarly adsorbed derivatives indicated that separation should be possible, a mixture of the two was chromatographed to determine how well the zones can be separated. In many instances where the plots of the individual derivatives overlapped slightly, visible interzones were observed when a mixture was developed. Although such chromatograms may not effect a complete separation, they are useful for purposes of identification. The pairs of derivatives which can be separated with the various developers are given in Table IV.

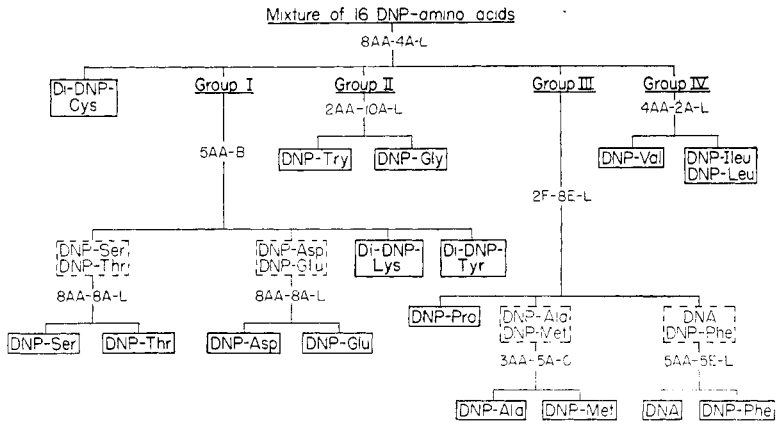

Figure 3. Systematic Scheme for Isolation and Identification of 16 Dinitrophenylamino Acids by Adsorption Chromatography on Silicic Acid–Celite

General Appearance and Behavior of Zones. With the developers containing acetic or formic acid the yellow zones formed by the 2,4-dinitrophenylamino acids on the silicic acid–Celite columns appear to be symmetrical with neither boundary sharp, especially on the outside of the column. Figure 1 shows the typical development behavior of the 2,4-dinitrophenyl derivatives. The lower edges of the zones usually move at a constant rate. The upper edges tend to move more slowly during the first 1 to 2 *V* ml. of developer than during subsequent development, when the rate of movement becomes constant.

On many chromatograms the zones appear to be considerably broader on the surface of the column than on the inside. Indeed, for some poorly separated mixtures a definite interzone may be found when the column is cut open, although there was little or no indication of separation on the surface. Downward coning of the zones on the inside is often observed and occasionally a zone is coned upward. As yet no satisfactory remedy has been found for such surface-spreading and coning phenomena.

The zones obtained with ligroin and cyclohexane developers are usually much narrower and more distinct than those with benzene.

The derivatives are more strongly adsorbed on 4 to 1 silicic acid–Celite than on 2 to 1 and in general the zones are sharper and hence the interzones are better. However, more time is required to run a 4 to 1 column, so that unless the separation on 2 to 1 is very poor, the higher ratio mixture has not been used.

THE SYSTEMATIC SCHEME

From the results of studies on the development behavior of the sixteen ether-soluble 2,4-dinitrophenylamino acids with a variety of developing solvents the systematic scheme shown in Figure 3 has been devised for the isolation and identification of each of these derivatives except 2,4-dinitrophenylisoleucine and 2,4-dinitrophenylleucine, which cannot be separated from each other. In general much simpler mixtures of the 2,4-dinitrophenylamino acids are obtained in studies on proteins and polypeptides, so that several steps in the scheme can often be eliminated.

Separation into Groups. As indicated in Figure 3, the unknown mixture to be examined is first developed with 8AA-4A-L in order to separate the mixture into groups. The amount of development required will depend on the mixture being examined. If there are members of Groups III and IV only, the development can be stopped while the zones are still on the column. However, if members of Groups I and II are present, it is necessary to wash some of the less strongly adsorbed zones into the filtrate.

Figure 4 shows the development behavior of a mixture of the 2,4-dinitrophenylamino acids when 3.5 *V* ml. of 8AA-4A-L are passed through the column and then development is continued with 10AA-5A-L. The relative positions of the derivatives within the groups are indicated by the heavy vertical bars. If only members of Groups II, III, and IV are present the development can be carried out with 8AA-4A-L alone, but when Group I is present, better separation of the more strongly adsorbed zones can be achieved by increasing the strength of the developer as shown. In cases where only a few members of a group are present in a mixture, some of the derivatives may be separated sufficiently to permit their isolation at this point. Incipient separation may often be observed between 2,4-dinitrophenylvaline and the 2,4-dinitrophenylisoleucine and 2,4-dinitrophenylleucine, between 2,4-dinitrophenylproline and the other members of Group III, and between the two members of Group II. After the development is completed and the column has been extruded, it is cut into sections containing the various groups or zones within the groups if they are separated sufficiently. The sections can then be eluted and prepared for rechromatographing with the appropriate developers.

Di-(2,4-dinitrophenyl)cystine and Group I. The relative positions of the members of Group I and of di-(2,4-dinitrophenyl)cystine with several developers are shown in Figure 2. Di-(2,4-dinitrophenyl)cystine is the most strongly adsorbed of the sixteen derivatives studied and can be separated readily from the others.

Group I is first developed with 5AA-B to separate its six members into four zones. After about 4 *V* ml. of this developer, the di-(2,4-dinitrophenyl)tyrosine zone is washed into the filtrate. After about 7 *V* ml. most of the di-(2,4-dinitrophenyl)lysine is also in the filtrate and two other zones are visible on the column. The lower zone consists of 2,4-dinitrophenylaspartic and 2,4-dinitrophenylglutamic acids and the upper zone contains 2,4-dinitrophenylthreonine and 2,4-dinitrophenylserine, which may be partially separated. Each of the latter two pairs can be resolved with 8AA-8A-L or 10AA-5A-L. The zones obtained on columns developed with 5AA-B are usually broader and the limits are more difficult to determine than with the ligroin or cyclohexane developers. Di-(2,4-dinitrophenyl)tyrosine forms an extremely faint zone with 5AA-B and may not be detected, so that it is wise to save the portion of the filtrate which might contain it and rechromatograph with 8AA-15E-L on a smaller column in order to see whether this derivative is present and to identify it.

The identity of the other members of this group can also be confirmed by development with 8AA-15E-L. Some 2,4-dinitrophenylpeptides and artifact zones adsorb in the same region as Group I, so that caution must be exercised in the identification of such zones.

Group II. 2,4-Dinitrophenyltryptophan and 2,4-dinitrophenylglycine can be separated at least partially with all the developers containing acetic acid and acetone in ligroin; incipient separation can be detected during the separation into groups. However, the best separation appears to be obtained with 2AA-10A-L. As Figure 2 indicates, the relative positions of

these two derivatives are reversed with developers containing ethyl acetate instead of acetone and this fact can be utilized in confirming the identification of the zones after they have been isolated, even though no separation is possible with the ethyl acetate developers.

Group III. From an examination of Figure 2 and Table IV it can be seen that the greatest number of separations in Group III can be obtained with 2F-8E-L. The only pair of derivatives which is not separated is 2,4-dinitrophenylalanine and 2,4-dinitrophenylmethionine. This pair is extremely difficult to resolve and as yet no developer has been found which gives a clean-cut separation. However, 3AA-5A-C or 2AA-6A-C gives two zones with a slightly colored interzone which is satisfactory for qualitative purposes. Better separations seem to be achieved on 4 to 1 silicic acid–Celite than on 2 to 1. For confirmation of the identity of these two derivatives, the zones can be chromatographed with 3% acetic acid in carbon disulfide, although the zones are pale with this developer and there is considerably more spreading of the zone on the surface than on the inside, so that when such a column is cut axially, the interzone is oval-shaped.

The 2,4-dinitrophenylproline derivative gives zones which are considerably paler than most of the 2,4-dinitrophenylamino acids and might easily be overlooked if present in small amounts.

Dinitroaniline (DNA), which may be present as an impurity, is adsorbed with 2,4-dinitrophenylphenylalanine with most of the developers, but the two can be separated with 5AA-5E-L. The identities of the zones isolated in Group III can be verified by their behavior with other solvent systems shown in Figure 2.

Group IV. Although a variety of developers containing formic or acetic acid and acetone or ethyl acetate in ligroin show qualitative separation of 2,4-dinitrophenylvaline from 2,4-dinitrophenylisoleucine and 2,4-dinitrophenylleucine, most of the interzones are faintly colored.

The best interzones have been obtained with 4AA-2A-L on 4 to 1 silicic acid–Celite. The relative positions of the three derivatives with this developer are shown in Figure 2. All the derivatives in this group give zones which are rather pale compared to most of the derivatives. All attempts to resolve mixtures of 2,4-dinitrophenylisoleucine and 2,4-dinitrophenylleucine have failed.

Dinitroaniline and Dinitrophenol. Because dinitroaniline and dinitrophenol may be encountered as by-products in the preparation and analysis of 2,4-dinitrophenylproteins, their behavior has also been studied with a number of developers used for the 2,4-dinitrophenylamino acids. Dinitroaniline is adsorbed with Group III and is included in the scheme. Dinitrophenol, which gives a nearly colorless zone, is considerably less strongly adsorbed than any of the 2,4-dinitrophenylamino acids and is washed off of the columns after 2 V ml. or less with all the developers used in the scheme. Dinitrofluorobenzene is also washed off the column rapidly.

Identification of Zones Isolated with Scheme. Diagrams like that shown in Figure 1 have been made of the development behavior of the 2,4-dinitrophenylamino acids with all the developers used in their isolation and identification. Tentative identification of the zones isolated in the scheme is made by comparing the development behavior of the unknown with the plots of the known 2,4-dinitrophenylamino acids under the same conditions.

There are great enough variations in the behavior of the derivatives with the different developers, so that if a zone behaves essentially the same as a known 2,4-dinitrophenylamino acid with several of the developers, its identity can be established with considerable certainty. Some confirmatory tests have been suggested in the discussion of the various groups.

The reproducibility of development behavior of the 2,4-dinitrophenyl derivatives is satisfactory in general, but several factors can cause some deviations from the expected behavior (see section on factors affecting development behavior). There are sometimes slight variations in the rate of movement of a given derivative when two parallel chromatograms which were run several weeks apart are compared. This is probably due to variations in the solvent batches or to the fact that the solvents have picked up some water. In instances where the behavior of a zone is similar to that of a known 2,4-dinitrophenylamino acid but where there are slight variations, the unknown and known samples should be compared under parallel conditions.

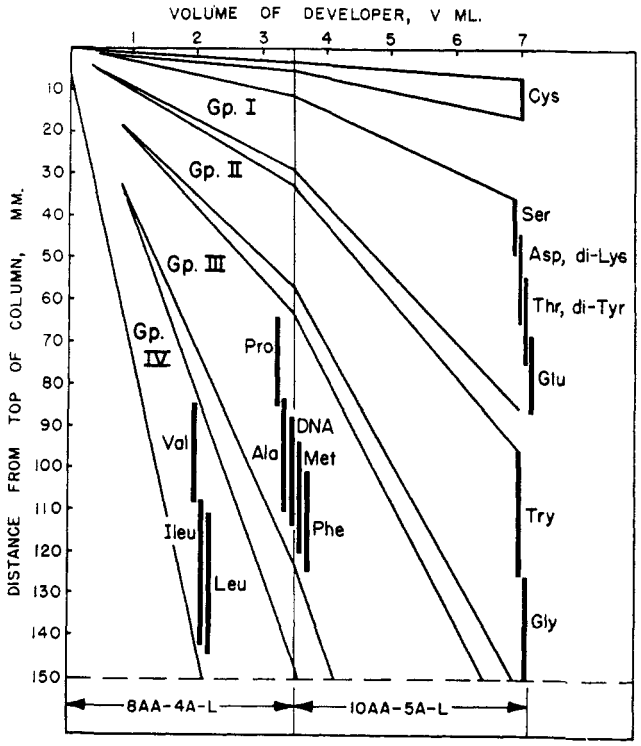

Figure 4. Development Behavior of Mixture of 16 Dinitrophenylamino Acids during Separation into Groups

Heavy bars indicate relative positions of individual derivatives within groups

Tests of Scheme on Different Batches of Silicic Acid. In order to determine whether the systematic scheme is widely applicable on a variety of samples of silicic acid, the developers used in the scheme were tested on twelve batches of commercially prepared silicic acid. All the samples were tested with a mixture of Groups II, III, and IV developed with 8AA-4A-L and with Group II developed with 2AA-10A-L. The three samples that gave poor results were not investigated further. The remaining tests were not run on all the satisfactory samples but only on a few which represented a cross section of the behavior observed in the first two tests. In cases where preliminary results indicated that the separations were rather poor on 2 to 1 silicic acid–Celite the tests were also run on 4 to 1 mixtures.

The various samples of silicic acid were graded on a relative basis for each separation that was tested. The results of the tests are summarized in Table V.

In general, samples designated A gave excellent separations with clear white interzones; those designated B gave good separations but the interzones were narrower or might be faintly colored; C indicates that the zones were definitely separated but that the interzones were not so wide or free of color as desired; those graded D showed either only slight indications of incipient separation or some indications of two zones inside the column but no visible interzone on the outside; if little or no separation was observed or if the zones were extremely poorly formed, the sample was graded F.

Table V. Comparison of Several Commercial Silicic Acids as Adsorbent for Chromatographic Separation of 2,4-Dinitrophenylamino Acids

Adsorbent		Sample: Groups II, III, IV	Group II	Group I	DNP-asp, -glu	Group III	DNP-ala, -met	DNP-val, -leu
		Developer: 8AA-4A-L	2AA-10A-L	5AA-B	8AA-8A-L	2F-8E-L	3AA-5A-C	4AA-2A-L
Merck 40446[a]	2:1[b]	B−	B−	C	C	B	...	...
	4:1	...	...	A	B	B−	B−	B−
Merck 40665[a]	2:1	B−	B	...	...	...	...	...
Merck 43243[a]	2:1	B	C−	...	...	...	...	...
	4:1	...	...	C	C+	...	...	...
Merck 42316	2:1	F	F	...	...	...	...	...
Mallinckrodt XXE-1[c]	2:1	B+	A	...	C+	...	...	...
	4:1	...	...	A−	...	B−	C	B+
Mallinckrodt XMH-1[c]	2:1	A−	A−	...	C−	...	...	...
	4:1	...	...	B+	C	B−	D	B
Mallinckrodt XXG-1[c]	2:1	A	B−	...	...	...	...	...
Mallinckrodt XAP	2:1	D	D	...	...	...	...	...
Mallinckrodt RCT-1[a]	2:1	B−	C+	...	...	...	...	...
	4:1	...	...	...	B	...	...	...
Baker A-288[a]	2:1	F	F	...	...	...	...	...
City Chemical[a]	2:1	B	C	...	D	D	...	...
	4:1	...	...	C+	B−	B−	D	B
General Chemical[a]	2:1	B	C+	...	B−	...	...	...
	4:1	...	...	...	B−	...	...	...

[a] Sample also studied by Trueblood and Malmberg for adsorptive properties (*22*).
[b] Ratio of silicic acid–Celite.
[c] Specially prepared for chromatographic purposes by manufacturer.

Silicic acid 40446, on which the scheme had been worked out, appears to be of intermediate quality, but even the poorest of the nine samples which were examined extensively gave satisfactory results if 4 to 1 silicic acid–Celite was used for most of the chromatograms. It had been anticipated that the two poorest samples (Merck 43216 and Baker A-288) might give unsatisfactory results, as these samples were much more granular than the others and the rate of flow of solvents through columns of these adsorbents was much greater than for the others. The third poor sample (Mallinckrodt XAP) also felt somewhat granular and had a rather fast flow rate, although not so great as the other two poor samples. It may be that after grinding and sifting to 100-mesh, these samples would also be suitable for chromatographic use.

The results of these tests indicate that it should not be difficult to find a commercial silicic acid which is suitable for use with the scheme. The three samples which were designated by the manufacturer as especially prepared for chromatographic purposes gave the best results. However, because of the slight variations in the adsorptive strengths of the various silicic acids, it will be necessary to determine the development behavior of each of the 2,4-dinitrophenylamino acids with each developer to be used in the method. It should be possible to determine whether a sample of silicic acid will be suitable by running a few exploratory tests. Samples that give poor results for one of the better separations, such as Group II with 2AA-10A-L, can be eliminated readily. If acceptable results are obtained with this mixture, the tests can be extended to some of the poorer separations such as that of 2,4-dinitrophenylaspartic and 2,4-dinitrophenylglutamic acids. If satisfactory separations are obtained here, the other portions of the scheme can then be tried.

In some instances more compact zones and better separations can be obtained by using a weaker developer than that prescribed in the scheme, but a greater volume of developer is required and hence the time required to run the chromatogram is increased. The degree of improvement attained with the weaker developer often does not warrant the additional time required. The sharpness of the zones and the interzones become progressively better as the strength of the developer is decreased.

Application of Scheme to Study of Lysozyme. The scheme has been successfully employed in determining that lysine is the terminal amino acid residue at the amino end of the lysozyme molecule (*6*) and by an extension of the method Schroeder has recently found that the sequence of the first four amino acid residues at this end of the polypeptide chain is lysyl-valyl-phenylalanyl-glycine (*18*).

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Correction

In the article on "Determination of Copper and Zinc in Soils or Plants" [ANAL. CHEM., **23**, 1861 (1951)] in the second column under the heading "Reagents" the first sentence describing the base electrolyte solution should read: "2.1 grams of sodium sulfite dissolved in 66 ml. of 0.1 *N* ammonium hydroxide, obtained by dilution of distilled ammonia with redistilled water."

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